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Topoisomerase II inhibition and high yield of endoreduplication induced by the flavonoids luteolin and quercetin

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Luteolin and quercetin are widely distributed plant flavonoids that possess a variety of chemical and biological activities, including free-radical scavenging and antioxidant activity. Recently, both flavonoids have been reported to inhibit DNA topoisomerases I and II (topo I and topo II), a property that, together with their ability to induce DNA and chromosome damage, has made them candidate anticancer compounds. In the present study, we confirmed that both compounds are topo II inhibitors by conducting a comparative study of their effect on topo II activity from Chinese hamster ovary AA8 cells. Because interference with the function of topo II to resolve DNA entanglement at the end of replication results in chromosome malsegregation at mitosis, we investigated whether luteolin and quercetin are effective in inducing endoreduplication in AA8 cells. Concentrations of luteolin and quercetin that inhibited topo II catalytic activity resulted in extraordinarily high yields of metaphases showing diplochromosomes. Given the established relationship of polyploidy with tumor development via aneuploidy and genetic instability, these results question the usefulness of luteolin and quercetin in cancer therapy.

Introduction

Flavonoids have a variety of biological and chemical properties. These compounds, widely distributed in the plant kingdom, are strong antioxidants (1,2) and have antimicrobial (3), anti-inflammatory/anti-allergic (4) antimutagenic (5), anticlastogenic (6) and anticarcinogenic properties (7,8). Due to their function as antioxidants, flavonoids are included, together with β-carotene and vitamins C and E, among the radioprotective molecules present in human diets that are rich in fruits and vegetables (9). These antioxidants may play an important role in scavenging free radicals, such as the highly reactive DNA-damaging hydroxyl radicals induced by ionizing radiation as a result of the radiolysis of water. Nevertheless, these reports of the beneficial effects of flavonoids are counterbalanced somewhat by studies indicating that flavonoids can induce DNA damage (10,11), mutations (12) and apoptosis (11,13).

Luteolin and quercetin are bioactive flavonoids that are closely related chemically (Figure 1). Both flavonoids protect against H_2O_2-induced DNA damage (14). Luteolin also protects cells from radiation-induced micronucleus formation (9,15), is toxic to various tumor cells, such as human leukaemia cells and pancreatic tumor cells (16,17), and induces DNA damage leading to apoptosis in human lung squamous carcinoma cells (11). Quercetin, on the other hand, is a DNA intercalating agent that is responsible for site-specific DNA cleavage in mammalian cells (18). However, evidence indicates that DNA damage by quercetin may occur via more than one mechanism, including mechanisms involving the participation of oxygen species (10,12).

Like the soy isoflavone genistein (13), other biflavonoids (19), and the plant flavonol fisetin (20), luteolin and quercetin are DNA topoisomerase (topo) inhibitors. Several reports have suggested that luteolin is an inhibitor of both topo I and topo II (21–23), poisoning both enzymes in the so-called 'cleavable complex'. Quercetin, on the other hand, poisons topo II (24) and topo I by preventing the DNA religation reaction after passage of the intact strand and relaxation (25). These results support the therapeutic potential of luteolin and quercetin as possible anticancer compounds.

In the present report, we have compared the ability of the two flavonoids, luteolin and quercetin, to inhibit topo II from Chinese hamster ovary AA8 cells. Given the unique role of the enzyme in chromosome segregation (26–28), we have studied the induction of endoreduplication by both flavonoids as a possible negative manifestation of the effects of these compounds on DNA topo II.

Materials and methods

Chemicals

Quercetin and luteolin were purchased from Sigma (St. Louis, MO). The chemicals were dissolved in dimethyl sulfoxide (DMSO) and added directly to the culture medium.

Cells and culture conditions

Chinese hamster ovary fibroblast cell line AA8 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The doubling time of AA8 cells is 13–14 h. Cells were grown as monolayers in McCoy's 5A medium (Bio Whittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and the antibiotics penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were cultured in a dark environment at 37°C in an atmosphere containing 5% CO_2. Regular testing indicated that the cell cultures were free of mycoplasma.

Preparation of nuclear extracts

Nuclear extracts were prepared from AA8 cells as described by Heartlein *et al*. (29). Cells, $\sim 1 \times 10^7$, were suspended in 1 ml of 0.32 M sucrose, 0.01 M Tris–HCl (pH 7.5), 0.05 M $MgCl_2$ and 1% Triton X-100 and thoroughly mixed with a vortex mixer to lyse the cells. Nuclear pellets were obtained by centrifugation at 1800 *g* for 5 min at 4°C. The nuclei were washed in 1 ml of nucleus wash buffer [5 mM potassium phosphate buffer (pH 7.5), 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM β-mercaptoethanol and 0.5 mM dithiothreitol (DTT)]. The nuclei were then pelleted as described above, resuspended in 50 μl of nucleus wash buffer, and 50 μl of 4×10^{-3} M EDTA were added. Following incubation at 0°C for 15 min, the nuclei were lysed by adding 100 μl of 2 M NaCl, 2×10^{-2} M Tris–HCl (pH 7.5), 10 μM β-mercaptoethanol and 1 mM PMSF. Following a 15 min incubation at 0°C, 50 μl of 18% polyethylene glycol (PEG-6000) in 1 M NaCl, 50 μM Tris–HCl (pH 7.5),

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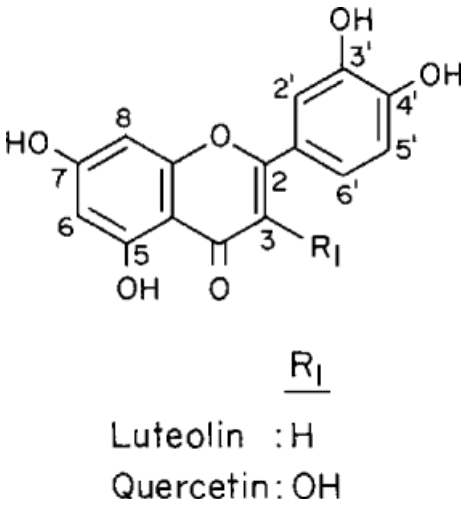

Fig. 1. Chemical structures of the flavonoids luteolin and quercetin.

10 mM β-mercaptoethanol and 1 mM PMSF were added. The suspension was incubated for a further 40 min at 0°C. The suspension then was centrifuged for 30 min at 11 200 *g* and 4°C, and the supernatant was collected. The total protein concentration in each extract (30) was determined in a Beckman DU-64 spectrophotometer by the Bio-Rad protein assay (Bio-Rad Laboratories, Munich Germany), and the extracts were stored at −80°C for no longer than 1 month.

Topo II activity in nuclear extracts

Topo II activity was assayed in nuclear extracts from untreated cells or extracts of cells incubated with 30–80 μM luteolin or quercetin using an assay kit (TopoGen, Columbus OH) that measured decatenation of kinetoplast DNA (kDNA). The reaction products were resolved using agarose gel electrophoresis of DNA. After incubation for 40 min at 37°C, the samples were loaded onto 1% agarose gels and subjected to electrophoresis for 2.5 h at 100 V. The gels were stained with 0.5 μg/ml ethidium bromide, destained for 30 min in distilled water, and photographed.

Induction of endoreduplication

Actively growing AA8 cells were cultured for 10 h in the presence of concentrations of luteolin or quercetin shown to inhibit topo II catalytic activity (see above). After treatment, the cultures were thoroughly washed and maintained in fresh medium for 18 h to allow them to recover. Cultures that did not receive any treatment served as controls. Colcemid (0.2 mM) was added for the final 2.5 h of cell culture in order to arrest metaphases. The flasks were gently shaken to dislodge the mitotic cells, which were collected by centrifugation, treated with 75 mM KCl for 2 min (hypotonic treatment), fixed in 3:1 methanol:acetic acid, and dropped onto clean glass microscope slides. The slides were stained with 3% Giemsa in phosphate buffer (pH 6.8), and mounted in DPX. Two thousand metaphases per culture were counted and classified as normal or containing diplochromosomes. All the experiments were carried out in triplicate.

Results

Effect of luteolin and quercetin on topo II catalytic activity

Topo II activity was measured as the ability of AA8 cell extracts to decatenate double-stranded catenated kDNA. Figure 2 shows that the topo II activity recovered in the nuclear extracts from AA8 cells was able to achieve efficient decatenation of the catenated DNA substrate as detected by the release of closed minicircles of double-stranded DNA. The addition of either flavonoid to the reaction resulted in a dose-dependent loss of decatenation capacity, as shown by a progressive increase in the amount of catenated DNA substrate remaining in the wells after the reactions (Figure 2). Nevertheless, the ability to inhibit topo II catalytic activity was somewhat different depending upon the compound tested, with a higher effectiveness of quercetin to fully inhibit decatenation by topo II of the catenated DNA substrate at lower doses than luteolin (see Figure 2, doses of 30 and 50 μM). This latter was also supported by data from image densitometry (not shown) and agrees with that reported by Constantinou *et al*. (31) for quercetin using a cell-free system to assess the effect on topo II catalytic activity (bacteriophage P4 knotted DNA) and cleavable complex stabilization (plasmid linearization assay), respectively.

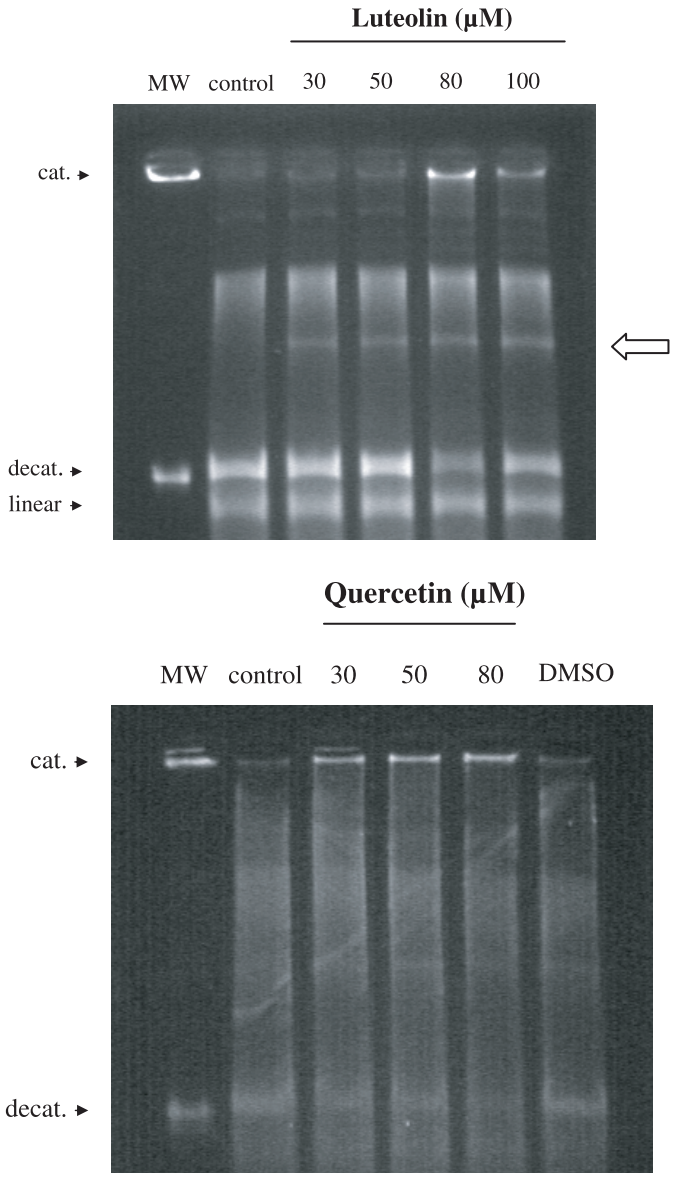

Fig. 2. Effect of luteolin and quercetin on DNA topo II activity. Topo activity was measured as the ability of an extract of Chinese hamster ovary AA8 cells to decatenate catenated kDNA (cat.) leading to the formation of double-stranded minicircles (decat.) or linear DNA. Also observe the appearance of a band located between those corresponding to catenated and decatenated DNA (arrow) which corresponds to partially digested intermediate forms (catenanes) that indicate a loss in topo II performance. Experiments were conducted in triplicate with similar results. MW, catenated and decatenated molecular weight markers. The lack of inhibition of topo II catalytic activity by the DMSO solvent is also shown along with quercetin.

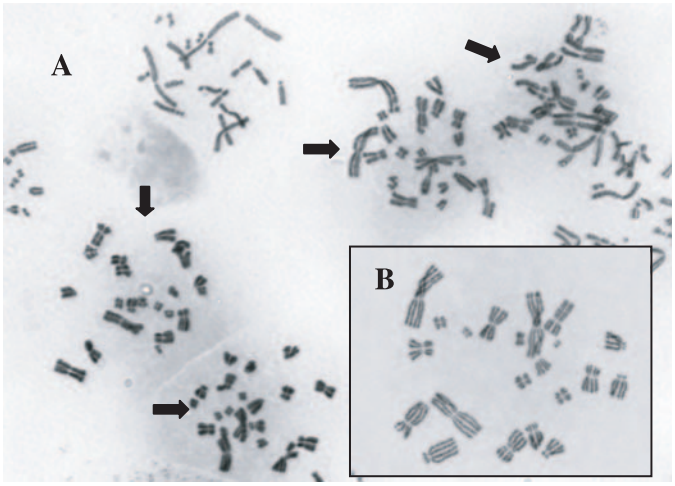

Fig. 3. Endoreduplication in Chinese hamster ovary AA8 cells treated with 80 μM luteolin. Low-magnification (500×) micrograph showing the efficient induction of endoreduplication as demonstrated by the number of metaphases containing diplochromosomes (arrows) (**A**) Higher magnification (1000×) of a metaphase showing diplochromosomes (**B**).

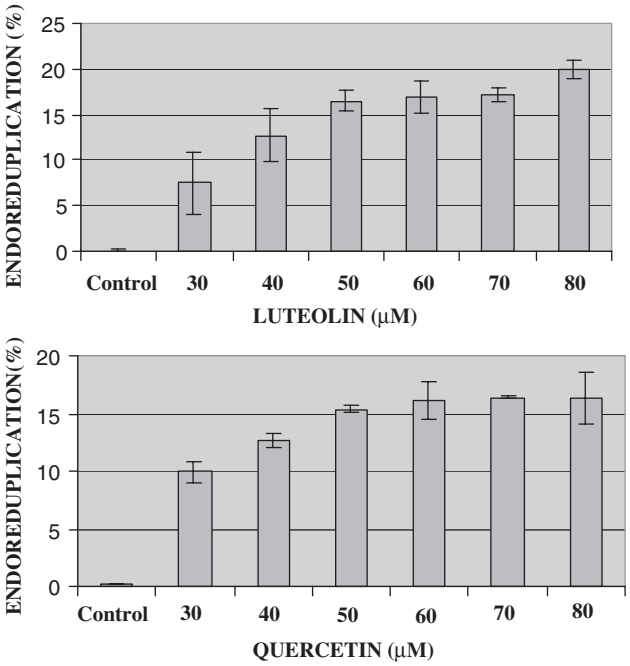

Fig. 4. Frequency of endoreduplication in Chinese hamster ovary AA8 cells treated with the flavonoids luteolin and quercetin. Test concentrations were selected on the basis of their catalytic inhibitory effect on DNA topo II. Endoreduplication (%) means percentage of mitoses showing diplochromosomes. Bars are the means ± the standard deviations (SDs) of data from three independent experiments.

Both flavonoids efficiently induce endoreduplication

Concentrations of luteolin and quercetin that efficiently inhibited topo II catalytic activity (Figure 2) were assayed for their ability to inhibit normal chromosome segregation during mitosis. The endpoint chosen to assess malsegregation leading to aberrant mitosis was endoreduplication (32,33), typically visible as metaphases made up of diplochromosomes (Figure 3).

AA8 cells were treated with either luteolin or quercetin for 10 h; the cells were then allowed to recover for an additional cell cycle during which endoreduplication (if any) might take place. As can be seen in Figure 4, endoreduplication was effectively induced by 30–80 μM quercetin and luteolin, while no clear differences in the percentage of metaphases showing diplochromosomes after treatment with either flavonoid was observed. Dose-dependent increases were induced at flavonoid concentrations up to 50 μM. At higher concentrations, increased endoreduplication frequency was still consistently observed, but the responses tended to plateau, possibly because of the induction of chromosome damage (e.g. multiply damaged metaphases, chromatid breaks and chromosome exchanges) that, in turn, might have caused cell cycle delay.

Discussion

In contrast to their antioxidative properties (see Introduction), the flavonoids quercetin and luteolin are also mutagenic and clastogenic (34,35) and induce DNA strand breaks (10,11,36). In addition, recent reports indicate that both luteolin and quercetin interact with the nuclear enzymes topo I and topo II (21–23,25). The interaction results in poisoning the enzyme in the ternary DNA–topoisomerase–flavonoid complex, the so-called 'cleavable complex'. In particular, luteolin has topo I inhibitory activity comparable to the potent topo I inhibitor, camptothecin; but unlike this alkaloid, luteolin binds to both the enzyme and to DNA itself (21).

After the passage of the replication fork during eukaryotic DNA synthesis, the double-stranded DNA molecules of sister chromatids inevitably become concatenated. Resolution of this DNA entanglement must occur as a pre-requisite for proper anaphase segregation (37). The nuclear enzyme DNA topo II is capable of breaking and resealing double-stranded DNA in a concerted fashion, and is the only known eukaryotic enzyme capable of decatenation (26,37). Studies with temperature-sensitive *Top 2* yeast mutants indicate that this enzyme has an essential role in decatenating sister chromatids before anaphase commences (38–40). Nevertheless, while topo II is required for chromosome condensation and chromatid segregation, it is clearly not needed for progression through the latter stages of mitosis, such as cytokinesis (39,41).

Endoreduplication in eukaryotes is a process that involves DNA amplification without corresponding cell division (26–28). While the molecular mechanisms responsible for endoreduplication are poorly understood, it is a useful endpoint for assessing failure in the proper decatenation of replicated chromosomes before mitosis (33,41,42). In this present study, we have assessed the ability of the flavonoids luteolin and quercetin to induce endoreduplication in cultured Chinese hamster ovary cells.

The present study confirms previous findings by us (43) and others (33) indicating that poisoning and catalytic inhibition of topo II result in different amounts of endoreduplication. Topo 'poisons' are chemicals that cause DNA strand breaks through stabilization of topo II covalently bound to DNA in the cleavable complex (44), while those classified as true catalytic inhibitors (45) are in general considered as not damaging DNA. Although direct evidence is still lacking, a likely explanation is that prevention of decatenation of replicated chromosomes by topo II leads to the subsequent failure to complete anaphasic segregation. Accordingly, any agent able to interfere with the enzyme function at any step of its catalytic

cycle would negatively affect proper anaphase segregation at mitosis. When this occurs, cells may follow either of two possible pathways: commitment to a genetic program that results in apoptotic cell death, or a pathway involving the skipping of anaphase, initiating a new cell cycle (endocycle), and forming polyploid or endoreduplicated cells. Cells taking the latter pathway undergo a further round of DNA replication, with the result that at the next mitosis they produce metaphases made up of diplochromosomes (26,27).

Endoreduplication by these flavonoid compounds has not been reported previously. The results of the present study show that both luteolin and quercetin are very strong inducers of endoreduplication as compared with a variety of chemicals previously tested in our laboratory (43,46–48) and are in good agreement with our recently proposed model for induced endoreduplication (28). In spite of the widely different levels of endoreduplication produced by different topo II inhibitors (28,43,47,48), the results are consistent with the proposal that a fully operative topo II is necessary for chromosome segregation before mitosis in mammalian cells, as shown earlier in yeast (38). As to a possible explanation for the extraordinarily elevated yield of endoreduplication observed by us for both flavonoids, though the unlikely explanation of a hypersensitivity of the AA8 Chinese hamster cell line cannot be as yet dismissed, given the lack of comparative data from other cell lines, the most likely hypothesis is that the dual action of these plant compounds on both DNA (intercalative etc.) and the enzyme itself (as a strong poison) results in a highly negative effect on topo II performance for chromosome segregation.

Luteolin and quercetin are regarded as potential anticancer agents. According to Liu (44), any compound that interferes with either topo I or topo II is a potential tool in cancer therapy. Luteolin was the most potent of 27 citrus flavonoids, including quercetin, in an assay of anti-proliferative activity against several tumor and normal cell lines (23). Other comparative studies have established luteolin to be a more specific and therapeutic agent than quercetin (24,49). Our present findings, however, indicate that the interaction of luteolin and quercetin with DNA topo II results in the malsegregation of chromosomes leading to a high yield of endoreduplication.

As recently reported for cisplatin (50), there is correlative evidence indicating that polyploid (usually tetraploid) cells are formed in a variety of pathological conditions, including during the development of some common tumors (51). A checkpoint has been described recently that arrests and eliminates polyploid cells (52). These observations suggest that the induction of polypoidy leading to apoptotic cell death may be a factor contributing to the efficacy of chemotherapeutic agents in tumor treatment. On the other hand, it also is possible that the genetic instability caused by the therapy results in the development of secondary cancers. Needless to say, this potential mechanism deserves special consideration.

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